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Seroresponses to Human Papillomavirus Types 16, 18, 31, 33, and 45 Virus-Like Particles in South African Women With Cervical Cancer and Cervical Intraepithelial Neoplasia

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The aim of the study was to determine the prevalence of antibodies to human papillomavirus (HPV) types 16, 18, 31, 33, and 45 in woman in Cape Town with cervical intraepithelial neoplasia (CIN) (n = 95), cervical cancer (n = 40), female blood donors (n = 95) and children (n = 110). The enzyme-linked immunosorbent assay (ELISA) made use of baculovirus synthesised HPV virus like particles (VLPs) as antigen. Antibodies to at least one HPV type were detected in sera from 75% of cancer patients, 71.6% of CIN patients, 44.2% of blood donors and 27.3% of children. Sera from 95 women with CIN were compared with age-matched female blood donors. There was a significant association of seropositivity to VLP-16 ($P = 0.006$) and VLP-45 ($P = 0.008$) with CIN compared with the blood donors. There was also a significant difference in the seropositivity of women with CIN to any of the five virus-like particle (VLP) types compared to the blood donors ($P = 0.0002$: OR = 3.2). Thirty-nine of sixty-nine (56.5%) women with CIN were found to be HPV-16 DNA positive. The average age of women in this group that were VLP-16 seropositive was 34 years and those found to be VLP-16 seronegative was 52 years of age. Antibodies to all five VLP types were detected in these populations, thus an ideal vaccine should induce protection from infection by a wide range of HPV types. ***J. Med. Virol. 60:403–410, 2000.***

KEY WORDS: HPV; serology; virus-like particles; cervical cancer

INTRODUCTION

Human papillomavirus (HPV) has been associated causally with cervical carcinogenesis [NIH consens statement, 1996]. The main HPV types associated with cervical cancer worldwide are HPV-16 (about 50%) followed by HPV-18 (14%), HPV-45 (8%) HPV-31 (5%) and HPV-33 (3%) [Bosch et al., 1995]. In South Africa, studies done in Cape Town and Durban indicate that HPV-16 is the predominant type associated with cervical disease [Williamson et al., 1989, 1994; Cooper et al., 1991].

With HPV vaccines under development, it will be imperative to determine the prevalent types within a region and which of those are associated with cervical disease. South Africa has an extremely high incidence of cervical cancer, especially among black women, where the life time risk is 1 in 34 and has age standardised incidence rate (ASIR) of 26.5 per 100,000 [Sitas et al., 1996]. The cervical cancer screening programs are inadequate and the best hope of controlling HPV infection and associated disease may therefore be a successful vaccination campaign. A serological assessment seemed appropriate as populations with high cervical cancer incidence have a higher HPV-16 antibody prevalence than those with a low incidence of cervical cancer [Strickler et al., 1999]. Also, seroreactivity to HPV virus-like particles (VLPs) is reported to be a better marker of risk of cervical cancer than HPV DNA [Nonnenmacher et al., 1995, 1996].

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VLPs formed from baculovirus expressed HPV L1 proteins were used [Rose et al., 1993]. These have been used to examine the immune response to HPV by enzyme-linked immunosorbent assay (ELISA). Seroreactivity to VLPs is associated with the presence of HPV DNA in the cervix [Kirnbauer et al., 1994]. This has validated the ELISA as 50–85% of patients with HPV DNA develop antibodies to that HPV type and the assay has been shown to detect serological evidence of past, present and persistent HPV infection [Kirnbauer et al., 1994; Wikström et al., 1995; Wideroff et al., 1995]. It has been demonstrated that antibodies to HPV-16 VLPs (VLP-16) and VLP-18 and VLP-33 antibodies are markers of sexual behavior [Dillner et al., 1996; Viscidi et al., 1997; Olsen et al., 1997]. VLP-16 antibodies may also be used to predict cervical disease [Dillner et al., 1995; Nonnenmacher et al., 1995; Carter et al., 1996], as VLP-16, VLP-18 and VLP-33 seroresponses are significantly associated with cervical cancer [Wang et al., 1997a]. The tests seem to be type-specific, as those with HPV DNA are more likely to have antibodies to that specific HPV type [Kirnbauer et al., 1994; Hamšiková et al., 1997; Wideroff, et al., 1999]. Even the phylogenetically closely related VLP-6 and VLP-11 have a type-specific response [Heim et al., 1995]. This assay was used previously to detect IgG antibodies to HPV-16 VLPs in 45% of cervical intraepithelial neoplasia (CIN) patients and 4.5% of children from Cape Town [Marais et al., 1997].

The aim of the study was to evaluate in the South African population, exposure to HPV types 16, 18, 31, 33, and 45 and to assess the relationship between cervical disease and seroreactivity to these HPV types. HPV types 16, 18, 31, 33, and 45 are associated with 80% of cervical cancers worldwide [Bosch et al., 1995] and there are no published reports on the seroresponses to 5 oncogenic virus-like particle (VLP) types. The data were analysed to assess whether there was serological cross reactivity between HPV types.

MATERIALS AND METHODS

Serum and Biopsy Samples

Serum and biopsy samples were drawn from patients attending a colposcopy clinic and from patients receiving in patient care at Groote Schuur hospital, Cape Town, South Africa during 1994 and 1997. Histology of biopsies taken from these women indicated that forty of these patients had cervical cancer (squamous) and 95 had CIN (80 were CIN3, 11 were CIN2 and 4 were CIN 1). As age-matched controls, sera were obtained from 95 female, blood donors at the Western Province Blood Transfusion Service in Cape Town. The prevalence of cervical disease amongst the donors was unknown but at the estimated prevalence of cervical cancer in South African women (ASIR of 22 per 100,000) [Sitas et al., 1996], none of the 95 would be expected to have cervical cancer. The CIN prevalence could be as high as 28 per 1000 [Leiman, 1987], or 2 of the 95 donors. The number of CIN patients per age group were 5 aged 21–25, 17 aged 26–30 years, 28 aged 31–35 years, 18 aged 36–40 years, 12 aged 41–45 years, 6 aged 46–50 years and 9 older than 50 years (mean age 37). It was not possible to obtain age-matched blood donor controls for statistical comparison with the patients with cervical cancer because of the advanced age of most of the members of this group (mean age 54 years). Cervical punch biopsies were obtained from all CIN and cervical cancer patients at the same time as the serum was collected, and used for HPV DNA determinations. Children's (110) sera from patients at a private pathologist in Cape Town were drawn for unrelated tests. The children were aged from 2–12 years. The CIN patients, blood donors and children had been part of a study of the age related seroresponses to HPV antigens [Marais et al., 1997]. The race of the individuals in the study was unknown.

HPV Serology

VLPs were produced in insect cells [Rose et al., 1993] from baculovirus-expressed recombinant L1 proteins of HPV-16, 18, 31, 33, and 45 and purified in CsCl gradients. These are all considered to be oncogenic HPV types, associated with cervical disease. To remove possible background seroreactivity, sera were also tested against bovine papillomavirus type 1 VLPs (BPV-VLP) that were prepared in the same way. Sera were tested for antibodies at a 1:20 dilution by ELISA, using 1 μg VLPs per well, as described previously [Marais et al., 1997]. Each ELISA plate included positive and negative control sera as internal standards for the assays. A serum was tested for each of the 5 VLP types at the same time, on two HPV-VLP wells and one adjacent BPV-VLP well. For each serum sample the OD value obtained on BPV-VLP coated wells was subtracted from the mean of two values obtained on the HPV-VLP coated wells.

ELISA cutoff values were determined using children's sera, as it has been shown that young children have low levels of antibodies to VLPs [Marais et al., 1997; Luxton et al., 1997; af Geijersstam et al., 1999]. A cutoff value was calculated for each VLP type using the mean children's OD value for that type, plus two standard deviations (mean + 2SD), after the elimination of outliers.

DNA Extraction and HPV Typing

DNA was extracted from biopsies as described previously [Williamson et al., 1994]. Standard precautions were taken to prevent contamination with amplicon DNA and PCR artifacts. The quality of the DNA was tested by the amplification of the CCR5 gene [Michael et al., 1997]. HPV-16 specific DNA was amplified as described by van den Brule et al. [1989].

Data Analysis

Data was analysed by χ^2 test using Epi Info Version 5 (Centres for Disease control, Epidemiology Program Office, Atlanta, Georgia). The significance level for assessing deviations from the tested hypothesis was $P =$

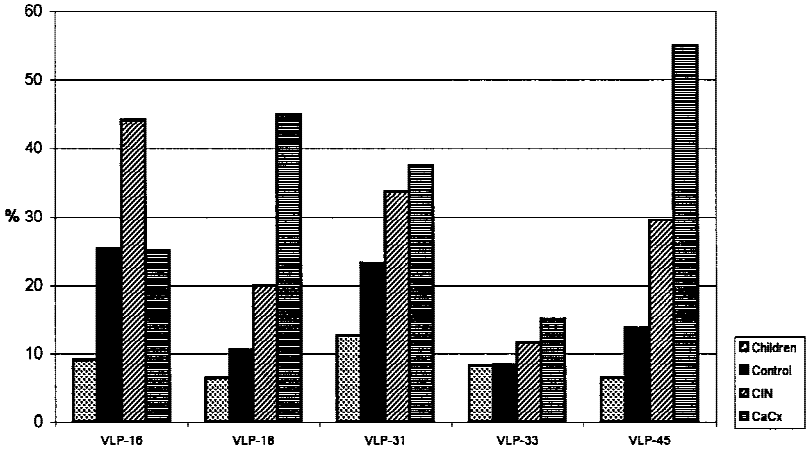

Fig. 1. The seroprevalence of CIN and cervical cancer patients, blood donor controls and children to HPV VLP types 16, 18, 31, 33 and 45.

0.05 for all tests. Scatterplot analysis was carried out using Microsoft Excel (Microsoft Corp., Redmond, WA).

RESULTS

HPV Serology

The prevalence of IgG antibodies to the five different VLP types as measured in cervical cancer and CIN patients, controls and children is represented in Figure 1. The frequency of seropositivity to each of the five HPV-VLP types was higher CIN patients than controls. With the exception of HPV-16, the seropositivity was even higher in women with cervical cancer than those with CIN. This was especially so for VLP-18 (45%) and VLP-45 (55%) (Fig.1). Statistical analysis showed a significant association with CIN compared with blood donor controls for VLP-16 ($P = 0.006$) and VLP-45 ($P = 0.008$), (Table I). Because of insufficient blood donors of advanced age for statistical comparison, it was not possible to determine the significance of the association of VLP antibodies in the cervical cancer patients with cervical disease.

Because so many individuals were positive to multiple VLP types, each group was examined with regard to their seronegative status, seropositivity to one VLP type and for multiple seropositivity (>1 type). Results are shown in Figure 2. The majority of children (72.7%) were found to be seronegative. In the adult groups, 75% of cervical cancer patients were seropositive for at least one VLP type. A significant number of CIN patients (71.6%) were seropositive to at least one VLP type compared to the control group (44.2%), (OR = 3.2, $P = 0.0002$). The majority of CIN patients had antibodies to either 1 or 2 types (54.7%) and 16.9% had antibodies to more than 2 VLP types. Of the patients with cervical cancer 27.5% had antibodies to >2 types. Table II shows the number of different VLP type combinations found in the blood donor, CIN and cervical cancer patient groups. Antibodies found most frequently in combination with one another in the same individual were to VLP-16 and -31 and VLP-18 and -45. Both were found in as many individuals (34), but in 15 women both of these antibody combinations (VLP-16 and -31 with VLP-18 and -45) were found in the same person. There were more women with dual VLP-18 and -45 antibody combinations (12) than dual VLP-16 and -31 combinations (8).

The high incidence of seropositivity to multiple VLP types raised the possibility of serological cross-reactivity between the different VLP types. Therefore, scatterplot analysis of OD values obtained for each serum against each VLP type was used to assess the seroreactivity of each of the VLP types with all of the other types. A weak correlation was only found between seropositivity to VLP-18 and VLP-45 (Fig. 3) in all the adult groups. For all other VLP types the R^2 value was <0.3 when compared with one another.

HPV DNA Analysis

To determine the prevalence of HPV infection in patients with cervical disease, DNA analysis was carried out on biopsies from 69 CIN and 24 patients with cervical cancer (Table III). All were positive for the CCR5 gene, indicating adequate DNA for analysis. HPV-16 DNA was detected in 39/69 (56.5%) of CIN cases and in 13/24 (54%) of cervical cancer cases. There was no statistical difference between the numbers of CIN patients who were HPV-16 DNA positive as well as VLP-16 antibody positive (22/39), and the CIN patients who were HPV-16 DNA positive and seronegative (17/39) ($P = 0.2$). In the cervical cancer group, only 2/13 (15%) of the women who were HPV-16 DNA positive were VLP-16 seropositive. Of the 11 patients with cervical cancer who were HPV-16 DNA negative, four (36.4%) were VLP-16 seropositive. In the CIN patient group, the average age of those positive for both HPV-16 DNA and VLP-16 antibodies was 34 years, whereas those who were DNA positive and VLP-16 seronegative had an average age of 52 years. Women with antibodies to VLP-16 who were negative for HPV 16 DNA had an average age of 37 years.

DISCUSSION

Antibodies to HPV VLP types 16, 18, 31, 33 and 45 were detected in all groups tested. There was an increase in seroprevalence to all the VLP types in the CIN patients compared to the blood donors. A similar

TABLE I. Seroprevalence of Antibodies to HPV-16, -18, -31, -33, -45 VLP in CIN Patients and Age Matched Blood Donor Controls

	CIN patients (n = 95)		Controls (n = 95)				
	n	%	n	%	Odds ratio	95% CI[a]	*P*-value*
VLP-16	42	(44.2)	24	(25.3)	2.34	1.12–4.55	0.006
VLP-18	19	(20)	10	(10.5)	2.13	0.87–5.27	0.069
VLP-31	32	(33.7)	22	(23.2)	1.69	0.85–3.36	0.107
VLP-33	11	(11.6)	8	(8.4)	1.42	0.50–4.11	0.468
VLP-45	28	(28.5)	13	(13.7)	2.64	1.20–5.86	0.008
Any VLP	68	(71.6)	42	(44.2)	3.18	1.83–6.73	0.0002

[a]I = confidence interval.
*χ^2 test for difference between two categories.

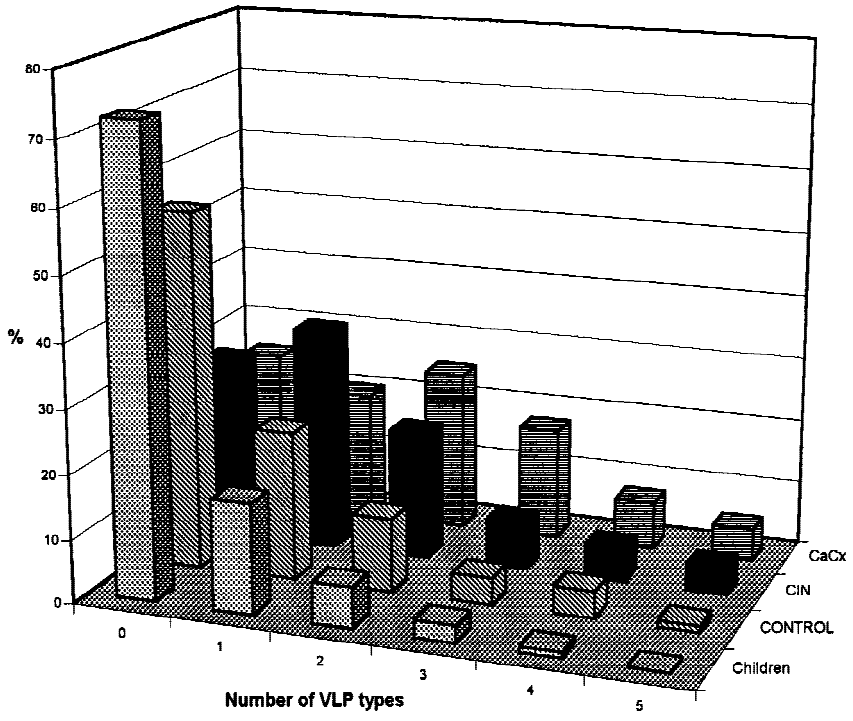

Fig. 2. The number of seronegative individuals per group and the seropositivity of the children, blood donor controls, CIN patients and cervical cancer patients to 1–5 VLP types.

increase was noted in the seroresponses of the cervical cancer patient group, compared with the CIN patients, except for the seropositivity to VLP-16. A reduction in the seroprevalence to VLP-16 antibodies in patients with cervical cancer compared to CIN patients has been described [Nonnenmacher et al., 1995] and seroprevalence decreased with the advancing stage of the invasive cancer [Park et al., 1998]. Disease association was found for seropositivity to VLP-16 ($P = 0.006$) and VLP-45 ($P = 0.008$) in the CIN group, compared with the blood donor control group. Comparing the latter two groups, there was also disease association with the combined seropositivity to any of the five VLP types tested ($P = 0.0002$, OR = 3.2). The association of VLP-16 antibodies with cervical disease is consistent with results from other studies worldwide [Chua et al., 1996; Kirnbauer et al., 1994; Dillner et al., 1995; Wideroff et al., 1995; Wang et al., 1997a]. This is the first published report of high seroprevalence to VLP-45 as well as disease association. This was unexpected, as a previous study found that the prevalence of HPV-45 DNA was low (1.5%) in cervical cancers from Cape Town [Williamson et al., 1994]. Bosch et al. [1995] reported that 12.4% of cervical cancers from Africa were associated with HPV-45.

The fact that no disease association ($P = 0.069$) was found in the CIN group for seropositivity to VLP-18 may be due to the high HPV 18 DNA prevalence found amongst the normal population in South Africa [Ramesar et al., 1996]. HPV serology would be expected to have the strongest predictive value for HPV associated disease in populations with a low HPV prevalence [Wang et al., 1997a]. Chua et al. [1996] assessing seropositivity to VLP-16, -18 and -33, reported that seropositivity to VLP-18 did not confer any measurable risk of developing CIN whereas antibodies to VLP-16 was indicative of a 3-fold risk ($P = 0.001$) of developing CIN. Wang et al. [1997a] showed an association of seropositivity to VLP-16, -18 and -33 with cervical cancer. It was not possible in the present study to determine

TABLE II. The Number of Times Single and Multiple Seropositivites Were Found in the Patient and Donor Groups

VLP type and type combinations	Donors (n = 95)	CIN patients (n = 95)	Cervical cancer patients (n = 40)	Total (n = 230)
16	10	17	1	28
18	4	1	1	6
31	6	10	3	19
33	1	2	0	3
45	1	3	3	7
16,18	1	1	0	2
16,18,31	0	0	1	1
16,18,31,33,45	1	4	2	7
16,18,31,45	2	4	2	8
16,18,33,45	0	0	1	1
16,18,45	1	3	1	5
16,31	3	5	0	8
16,31,33	1	2	0	3
16,31,33,45	2	1	0	3
16,31,45	1	1	2	4
16,33	1	1	0	2
16,45	1	3	0	4
18,31	0	1	1	2
18,31,45	0	0	1	1
18,33,45	1	0	1	2
18,45	0	5	7	12
31,33,45	0	1	0	1
31,33	2	0	1	3
31,45	3	3	2	8
Total	42/95 (44%)	68/95 (72%)	30/40 (75%)	140/230 (61%)

the association of VLP antibodies in the cervical cancer patients with disease.

The combined seropositivity to any VLP type was significantly higher ($P = 0.0002$) in the CIN patients compared with the blood donors. This would not be useful as a marker of disease in individual women, however, as 44% of the control group has antibodies to at least one of the VLP indicating that HPV infection is common in the general population and infection per se does not predict progression to disease. Indeed, 27.3% of the children were also positive to at least one VLP type. This study is the first published report of antibodies in children to 5 oncogenic HPV VLP types.

A decrease in seropositivity to VLP-16 with age has been reported [Marais et al 1997]. The present study compared the HPV-16 DNA status with serology. There was no correlation between positive HPV-16 DNA status and seropositivity to VLP-16. Nonnenmacher et al. [1996], Wang et al. [1997a], Carter et al. [1996] and Park et al. [1998] described a similar lack of correlation between serology and DNA status. It is therefore unlikely that comparison of DNA status and serology for all 5 different VLP types would indicate whether there is any cross reactivity between types. In the CIN patient group, the average age of those HPV-16 DNA and VLP-16 antibody positive was 34 years, whereas those who were DNA positive and VLP-16 antibody negative had an average age of 52 years. Most of cervical cancer patients (11/13) who were HPV-16 DNA positive were VLP-16 antibody negative. This indicates a gradual decline in HPV-16 VLP antibodies with time in women with high-grade lesions and that in most women with HPV-16 associated cancers VLP-16 antibodies were below the level detected by the ELISA. In cervical cancers there is no active virus production and therefore no detectable L1 expression [Bohm et al., 1993]. The lack of L1 to stimulate the immune system in lesions may lead to a drop in antibody titres. Carter et al. [1996] found no reduction in VLP-16 antibody titres in college students followed up for four years after seroconversion. Af Geijersstam et al. [1998] found no change in HPV-16 seropositivity in women between their first and second pregnancy. In our study, however, the women are much older and the time that lapsed since the initial HPV infections could be over 30 years. The gradual decline in HPV-16 antibodies over time does not explain the high seroprevalence in the cervical cancer and CIN groups to the other high-risk VLP types. Antibodies to the VLP-18, -31, -33 and -45 implies a more recent cervical exposure to these VLP types, or concurrent productive infection at a site other than the cervix. Another possible explanation is that HPV-16 is less immunogenic than the other genital HPV types [Rose, unpublished data].

In this study, seropositivity to multiple HPV types was common and is substantiated by a similar report in Czech women [Tachezy et al., 1999]. Our study was not able to confirm whether multiple seroprevalence was an indication of cross-reactivity between HPV types 16, 18, 31, 33, and 45 or infection with multiple types, or a combination of these two possibilities. This will only be confirmed in a longitudinal study assessing the relationship between multiple viral exposure and seroprevalence. A comparison of donor and patients' OD values by scatterplot analysis only indicated possible cross reactivity between VLP-18 and VLP-45. The as-

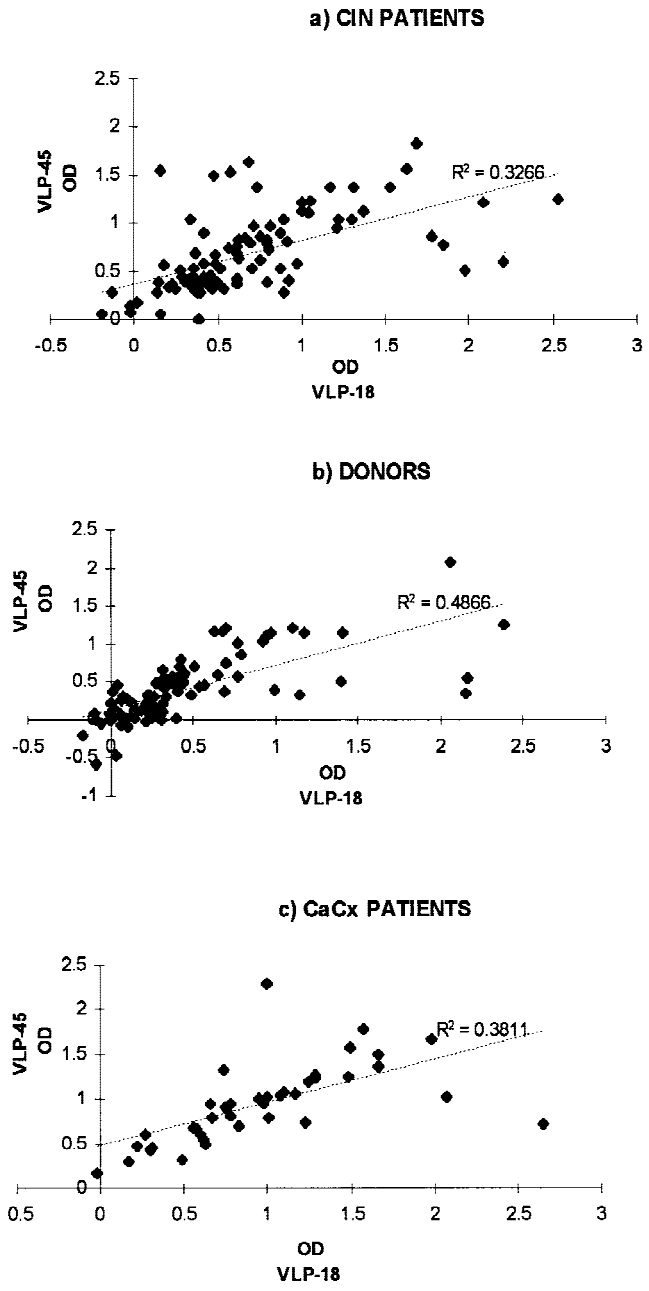

Fig. 3. The comparison by scatterplot analysis of the seroreactivity to VLP-18 and VLP-45 in (a) CIN patients; (b) blood donor controls; and (c) cervical cancer patients. R = correlation coefficient.

sessment of the number of single and multiple anti-VLP sero-combinations in adult the groups showed little cross reactivity between the VLP types, with the exception possibly of VLP-16 and -31 and VLP-18 and -45. A greater number of women showed a seroprevalence to these two combinations than any others, that is consistent with their phylogenetic relatedness, but many women were also seropositive to only one of the five types. Seropositivity to multiple HPV types implies that infections by these types are in addition to that by HPV-16 and that infection by one type is not protective of infection by other types. It was shown recently that there is no increased risk of cervical cancer among women seropositive to VLP-16 in combination with VLP-11 or -6 or -18 or -33 [Loustarinen et al., 1999]. That study described an antagonistic interaction between these dual infections, that could be explained by cross protective HPV-specific cell-mediated immunity. In the present study, the CIN and especially the cervical cancer patients were more likely to be seropositive to more than one VLP type, compared with the other groups studied. Only in these patient groups were antibodies to VLP-18 and VLP-45 individually and in combination found most prevalent. This could indicate a broadening of the antibody response with time, to react to multiple epitopes, that has been suggested in accordance with the immunodominance theory [Wang et al., 1997b]. Early infections stimulate a single dominant epitope, but upon prolonged antigenic exposure cross-reactive antibodies to minor epitopes are introduced. Women were seropositive to different combinations of VLP, indicating that if there is a broadening of the epitopes recognised, there must be different epitopes recognised in different women. Whether this is of pathological significance in the development of HPV associated disease is unknown.

More of the patients with cervical cancer also had antibodies to multiple HPV types, especially if over 40 years. This implies that these women are more susceptible to infection with multiple HPV types, exposed to more types, more likely to have persistent infection, or more likely to mount a measurable antibody response, with a broadening of the antibody response to include cross-reactive antibodies. Other studies have reported that women with multiple seroprevalence have an elevated OR with increased lifetime number of sexual partners [Wideroff, et al., 1999], and this increased OR could be reduced if adjusted for life time number of sexual partners [Dillner et al., 1996]. This implies multiple seroprevalence may be related to high-risk sexual behaviour. We do not have details on the number of sexual partners and future studies should control for this variable.

Knowledge of the HPV status of a population is necessary for the design of preventative measures. This study has demonstrated antibodies all five VLP types in the groups assessed and antibodies to VLP-16 and VLP-45 to be associated with CIN. Patients with cervical cancer had high seroprevalence levels to VLP-18, -31 and -45. Whether the seroprevalence of types 18, 31 and 45 is associated with the high incidence of cervical cancer in South Africa remains to be evaluated with appropriate comparative studies. This study emphasises the need for a multivalent HPV vaccine in the southern African region, as although there might be some serological cross reactivity, there does not seem to be appreciable cross protection between VLP types with many women, especially those with cervical cancer, displaying antibodies to multiple types.

TABLE III. Results of the HPV-16 DNA Analysis of Biopsies From Women With CIN and Cervical Cancer and the Comparison With Their VLP-16 Antibody Status

	HPV-16 DNA positive	HPV-16 DNA negative	Total
CIN			
Seropositive to VLP-16	22/69	9/69	31/69 (44.9%)
Seronegative to VLP-16	17/69	21/69	38/69 (55.1%)
Total	39/69 (56.5%)	30/69 (43.5%)	
Cervical cancer			
Seropositive to VLP-16	2/24	4/24	6/24 (25%)
Seronegative to VLP-16	11/24	7/24	18/24 (75%)
Total	13/24 (54.1%)	11/24 (45.8%)	

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